# NEISSERIA LACTOFERRIN BINDING PROTEIN

## FIELD OF INVENTION

This invention relates to newly identified polynucleotides, polypeptides encoded by them and to the use of such polynucleotides and polypeptides, and to their production. More particularly, the polynucleotides and polypeptides of the present invention relate to the neisserial outer-membrane protein (OMP) family, and in particular to lactoferrin-binding protein B (LbpB). Furthermore, the invention relates to the therapeutic use of LbpB, such as for vaccination against neisserial disease.

## BACKGROUND OF THE INVENTION

Meningitis is either of bacterial or viral origin, the bacterial form being by far the most severe. The bacteria mainly responsible are *Neisseria meningitidis, Haemophilus influenzae* and *Streptococcus pneumoniae*. Since the launch of a conjugate vaccine against *H. influenzae* type B (Hib), and its integration into routine infant vaccination, *N. meningitidis* is taking over as the leading cause of meningitis throughout the world, there being an estimated 2,600 cases per year in the USA alone.

The species *N. meningitidis* is subdivided into 13 serogroups according to the composition of the capsular polysaccharides. In addition, each serogroup is sub-classified into serotypes, subtypes, and immunotypes on the basis of other components of the bacteria. Three serogroups (A, B, and C) account for more than 90 % of cases of meningitis, and in developed, industrial nations, serogroup B is responsible for 50 to 80 % of cases.

Effective vaccines based on capsular polysaccharides exist to prevent meningitis caused by *N. meningitidis* serogroups A and C. The serogroup C polysaccharide vaccines do not produce a protective effect in children less than 2 years of age (the age range where there is the greatest risk of developing meningitis), however this drawback may be overcome by conjugating these polysaccharides to a carrier protein. Conjugation has the additional advantage of inducing an immunological memory against the antigen.

In contrast, the polysaccharide of *N. meningitidis* serogroup B displays little or no immunogenicity in man, irrespective of whether or not it is in a conjugated form. It would

therefore be highly desirable to obtain a vaccine against neisserial disease induced by *N. meningitidis* (in particular of serogroup B) other than a polysaccharide-based vaccine.

A promising class of vaccine candidates are those using the outer membrane proteins (OMPs) of *N. meningitidis,* because they may provide antigens that are immunogenic and accessible to the human immune response. The OMPs responsible for the uptake of iron into the cell are particularly promising.

Iron is an essential nutrient for most bacteria. In the extracellular compartments of the human body iron is complexed mainly to transferrin in serum and to lactoferrin on mucosal surfaces (Finkelstein *et al.*, 1983), with negligible amounts in the free form. Therefore, efficient iron acquisition is an important virulence factor for pathogenic bacteria. As regards *N. meningitidis* in particular (a strict pathogen of man), its iron requirements are met by using receptors for human iron-chelating proteins, such as transferrin or lactoferrin, which enable the cell to bind these proteins and thereafter to take up the iron needed for its growth. The synthesis of these receptor proteins is induced when the bacteria sense iron limitation.

The receptor proteins involved in the uptake of iron from transferrin, TbpA and TbpB (Cornelissen *et al.*, 1992; Legrain *et al.*, 1993; Anderson *et al.* 1994) and from lactoferrin binding protein A (LbpA) (Pettersson *et al.*, 1993; 1994b; Biswas and Sparling, 1995) have been cloned and sequenced. The transferrin-binding receptor proteins form a complex in the outer membrane. In *N. meningitidis*, both TbpA and TbpB seem to be necessary for iron transportation (Irwin *et al.*, 1993). TbpA is an integral membrane protein, whereas TbpB is a lipoprotein and is anchored to the membrane only with its lipid moiety. The current model for the mechanism of the receptor proposes that iron-loaded transferrin binds to the receptor complex. In this complex, the TbpB protein discriminates between ferrated- and apo-transferrin. Binding of transferrin results in the conformational change in the receptor, which releases iron from transferrin and opens a gated pore in TbpA, and iron can be transported accross the outer membrane (Cornelissen and Sparling, 1994; 1996).

The lactoferrin receptor is also thought to be an important virulence factor of *N. meningitidis*. The main site of entry into the human body is the nasopharynx, where lactoferrin is the main iron source. Furthermore, preliminary reports show that lactoferrin is able to cross the blood-brain barrier in acute inflammation (Gschwentner *et al.*, 1997). It is

possible that lactoferrin is also an important iron source for the meningococci at a later stage of infection, when the bacteria have reached the meninges. By using an affinity isolation procedure, a single lactoferrin-binding protein was originally identified (Schryvers and Morris, 1988). The structural gene for this receptor, designated LbpA, has been characterised (Pettersson *et al.*, 1993; 1994b; Biswas and Sparling, 1995) and a topology model for the protein in the outer membrane has been proposed (Pettersson *et al.*, 1994a). The protein shows homology to TbpA. In addition, part of a possible open reading frame was identified upstream of the *lbpA* gene, and the deduced amino acid sequence showed homology to TbpB (Pettersson *et al.*, 1994a).

TbpB and other purified meningococcal OMPs have been the subject of previous patent applications with respect to their use as vaccines against *N. meningitidis* (e.g. TbpB, WO 9307172; 22 kDa surface protein, WO 9629412; haemoglobin receptor, WO 9612020; porin protein, WO 9503413; pilin proteins, WO 9408013; 64 kDa OMP, EP 474313-B1).

There is a need for identification and characterization of further members of the OMP family which can play a role in preventing, ameliorating or correcting dysfunctions or diseases, including, but not limited to, neisserial disease (for example meningitis).

Antibodies against LbpA do not seem to be bactericidal and it may therefore be of limited use as a vaccine candidate (Pettersson *et al.*, 1993).

This invention identifies and characterises another lactoferrin-binding receptor protein, lactoferrin binding protein B (LbpB), its role in the utilisation of iron from lactoferrin, and its therapeutic uses.

There are several advantages LbpB has over the other OMP vaccine candidates. Firstly, in the blood-borne stage of meningococcal disease human lactoferrin is essential to the organism as it has a 300 fold greater affinity for binding iron than human transferrin, and hence the use of lactoferrin as an iron source is essential to the organism. Secondly, lactoferrin has a known antibacterial effect, and its concentration in the blood increases upon infection. It is also therefore important for the organism to bind human lactoferrin as a way of gaining some resistance to this effect. Lastly, human lactoferrin is the main source of iron to *N. meningitidis* at the place of entry of the bacteria to the human body (the nasopharynx).

The significance of these advantages is that LbpB antigens would be likely to be expressed at the cell surface in the vast majority of meningococci in the body, that the cell-surface domain of LbpB is likely to be very conserved because it must effectively bind lactoferrin, and that an immune response directed against the LbpB antigen may not only stop any infection of meningococcus in the blood, it may also stop even the carriage of the organism in the nasopharynx.

## SUMMARY OF THE INVENTION

In one aspect, the invention relates to LbpB polypeptides and recombinant materials and methods for their production. Another aspect of the invention relates to methods for using such LbpB polypeptides and polynucleotides. Such uses include the prevention and treatment of neisserial disease (for example meningitis), among others. In still another aspect, the invention relates to diagnostic assays for detecting diseases associated with the presence of LbpB.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Fig. 1.** Western blot analysis of proteins from whole cells grown under iron limitation. Lanes 1 and 5, strain BNCV; lanes 2 and 6, *lbpA* mutant CE1452; lanes 3 and 7, *lbpB* mutant CE1454; lanes 4 and 8, *lbpAB* mutant CE1402. Antisera used were directed against synthetic peptides, based on the LbpB sequence. Lanes 1 to 4, serum 17-3 against peptide C1. Lanes 5 to 8, serum 19-1 against peptide E1. The positions of molecular size standards are indicated at the right in thousands. The LbpB protein is marked with an arrow at the left.

**Fig. 2.** Restriction map of the DNA fragments containing the *lbpB* and *lbpA* genes of strain BNCV. The inserts in the different recombinant plasmids and the PCR product (PCR) are shown as open boxes. Plasmids pAM23 and pAM1 contain fragments of the *lbpBA* locus that were characterised previously (Pettersson *et al.* 1993, 1994a). Open reading frames are marked with heavy arrows. Probes, used for screening the library or for Southern blotting, are shown above the open reading frames. Positions of the primers used for PCR amplifications are shown underneath the open reading frames. The insertion

site of the kanamycin resistance box in pAM6K is shown by an open triangle. The erythromycin resistance box in pAM23E is shown by a closed triangle.

**Fig. 3.** Alignment of proteins LbpB of strain BNCV and TbpB of strain B16B6. Identical amino acids are marked by dashes. The numbers to the right indicate the positions of amino acids. Gaps (-) were introduced to achieve optimal alignment. Peptides used to immunise mice are indicated above the sequence of LbpB. Two stretches, rich in negatively charged residues are underlined. The putative signal peptidase II cleavage site is shown with an arrow above the sequence.

**Fig. 4.** Sequence of the promoter area upstream of *lbpB*. The translation initiation site (ATG) is marked in bold. The ribosome binding site, and the putative -10 and -35 boxes are underlined (thick line and thin lines respectively). The putative Fur box is boxed.

**Fig. 5.** Western blot analysis of proteins from whole cells grown in TSB medium (lanes 1, 3, 5, and 7) or in TSB with EDDA (lanes 2, 4, 6 and 8). Antibodies used were monoclonals mn98k1 and mn98k2 directed against LbpA (Panel A) or antiserum 17-3 against LbpB peptide C1 (Panel B). Lanes 1 and 2, strain BNCV; lanes 3 and 4, *lbpA* mutant CE1452; lanes 5 and 6, *lbpB* mutant CE1454; lanes 7 and 8, *lbpAB* mutant CE1402.

**Fig. 6.** A. Western blot analysis of proteins from outer membranes of the meningococcal strain BNCV grown under iron limitation. The outer membrane proteins were electrophoresed under non-denaturing conditions and the LbpB protein was detected with the serum directed against the synthetic peptide A1. Lanes 1 and 2 show samples incubated at 0°C and 95°C, respectively prior to electrophoresis. The positions of the molecular size standards are indicated at the right in thousands. B. Lactoferrin binding assay on a Western blot with proteins from outer membrane complexes of the meningococcal strain BNCV grown under iron limitation. The proteins from the outer membrane complexes were electrophoresed under non-denaturing conditions and the blot

was incubated with peroxidase-coupled human lactoferrin. Lanes 1 to 3 show samples incubated at 0°C, 37°C, and 95°C, respectively prior to electrophoresis. The positions of the molecular size standards are indicated at the right in thousands.

**Fig. 7.** Binding of lactoferrin to *lbp* mutants in whole-cell ELISA-type assay. Strains, indicated at the right, were coated to the wells. Lactoferrin was added in concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, and 0 ng/ml (rows 1-8 respectively). Lactoferrin bound to the cells was detected with peroxidase-conjugated lactoferrin-specific antiserum.

**Fig. 8.** Plate feeding assays of strains with recombinant human lactoferrin. Only the relevant part of the plate is shown. Cells of the strains indicated at the right were plated on iron-restricted plates. Growth stimulation by drops of lactoferrin at the concentrations indicated was monitored after overnight growth. Arrows show the position of the drops in Panel B. In this experiment, 11 % iron-saturated lactoferrin was used.

**Fig. 9.** Alignment of the LbpB proteins from five meningococcal strains. The alignment was performed with the CLUSTAL program (PC Gene, IntelliGenetics), and optimized by hand. Numbers to the right indicate the positions of the amino acids. Gaps (-) were introduced to achieve optimal alignment. Positions where all five sequences are identical, are marked with *.

**Fig. 10. A.** Restriction maps of the relevant parts of pJP29 (Bosch *et al.*, 1986), pAM31 and pAM32. Only the inserts are shown. The vector is pACYC184. pJP29 contains the *phoE* gene (in light grey) behind its own promoter. The promoter and flanking sequences are in white. The *Pst*I- site is at the border of the sequences corresponding to the signal sequence and the mature part of the PhoE protein. pAM31 contains, from left to right: The *phoE* promoter (in white), and a recombinant gene encoding the signal sequence of PhoE (light grey) and the mature LbpB (black). DNA fragments corresponding to the N terminus of LbpA (dark grey), the C terminus of PhoE (light grey), and flanking sequences (white) are present as well. pAM32 was constructed

from pAM31 by inserting a linker (striped box), encoding a His-tag and a Factor Xa cleavage site, into the *Pst*I- site of pAM31. See Example 8 for details about the construction of pAM31 and pAM32. The restriction sites on pAM32 are in brackets, because they are lost during the cloning procedure.

**B.** The amino acid sequences of the recombinant LbpB construct (underneath) as compared with the wild-type LbpB sequence (on top). Only the last and first amino acid residues of the LbpB/PhoE signal sequences and mature LbpB, respectively, are shown. The His-tag and the Factor Xa cleavage site are shown entirely. The leader peptidase I and II (LPaseI and II, respectively) and Factor Xa cleavage sites are shown by arrows.

**Fig. 11.** PAGE of the purified recombinant LbpB protein. Lanes 1 and 2 show samples incubated at 0°C and 100°C, respectively, before electrophoresis. The positions of molecular weight standards are indicated on the right in kDa.

**Fig. 12.** Western blot with folded (lanes 1, 3, 5, 7, and 9) and denatured (lanes 2, 4, 6, 8, and 10) recombinant LbpB with five human convalescent sera. Lanes 1 and 2, serum 69; lanes 3 and 4, serum 262439; lanes 5 and 6, serum 262532; lanes 7 and 8, serum 263017, lanes 9 and 10, serum 330. The positions of molecular size standards are indicated at the right in kilodaltons. Arrows at the left indicate the positions of denatured (dLbpB) and folded LbpB (fLbpB).

**Fig. 13.** Results of the anti Whole Cell and anti-LbpB ELISA (Table 7) performed as described in Example 10. A. anti-LbpB response in mice immunized with either LbpB or *N. meningitidis* strain BNCV whole cells. A control immunization was carried out with PBS solution. B. anti Whole Cell (strain BNCV grown in iron deficient conditions) response in mice immunized with either LbpB or *N. meningitidis* strain BNCV whole cells. A control immunization was carried out with PBS solution. C. anti Whole Cell (strain H44/76 grown in iron deficient conditions) response in mice immunized with either LbpB or *N. meningitidis* strain BNCV whole cells. A control immunization was carried out with PBS solution.

**Fig. 14.** Results of the bactericidal activities (Table 8) of anti Whole Cell and anti strain BNCV LbpB sera performed as described in Example 10. **A.** Bactericidal titer against *N. meningitidis* strain H44/76 (grown in iron-rich conditions). **B.** Bactericidal titer against *N. meningitidis* strain H44/76 (grown in iron-depleted conditions).

## DESCRIPTION OF THE INVENTION

### Definitions

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"LbpB" refers generally to a polypeptide, preferably a lipoprotein, having the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, or 10, or an allelic variant thereof.

"LbpB activity or LbpB polypeptide activity" or "biological activity of the LbpB or LbpB polypeptide" refers to the metabolic or physiologic function of said LbpB including similar activities. Specifically, the LbpB activity is the ability to bind to human lactoferrin. This activity of LbpB can be tested using the method described in Example 6. Also included in this definition are antigenic and immunogenic activities of said LbpB. This antigenicity can best be tested using the immunoblot method described in Example 9, preferably using polyclonal sera against LbpB of meningococcal strain BNCV as described in Example 10A. The immunogenicity can best be tested by measuring antibody responses (using polyclonal sera generated against the variant) in ELISA using purified LbpB from meningococcal strain BNCV, as described in Example 10B.

"*lbpB* gene" refers to a polynucleotide having the nucleotide sequence 100-2274 set forth in SEQ ID NO:1, or the complete nucleotide sequence set forth in SEQ ID NO: 3, 5, 7, or 9, or allelic variants thereof and/or their complements.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of a Fab or other immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide

naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given

polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslational natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Meth Enzymol* (1990) 182:626-646 and Rattan *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci* (1992) 663:48-62.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential biological properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and,

in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant (for instance, SEQ ID NO:3, 5, 7 or 9 are variants of the *lbpB* polynucleotide of SEQ ID NO:1; and SEQ ID NO:4, 6, 8 or 10 are variants of the LbpB polypeptide of SEQ ID NO:2), or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis. Variants should retain one or more of the biological activities of the LbpB polypeptide. They should either be capable of binding human lactoferrin (preferably as described in the test for this activity in Example 6), or have similar antigenic or immunogenic activities as LbpB. The antigenicity can best be tested using the immunoblot method described in Example 9, preferably using polyclonal sera against LbpB of meningococcal strain BNCV as described in Example 10A. The immunogenicity can best be tested by measuring antibody responses (using polyclonal sera generated against the variant) in ELISA using purified LbpB from meningococcal strain BNCV, as described in Example 10B. Preferably, a variant would retain all of the above biological activities.

"Identity" is a measure of the identity of nucleotide sequences or amino acid sequences. In general, the sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques. See, e.g.: (COMPUTATIONAL MOLECULAR BIOLOGY, Lesk, A.M., ed., Oxford University Press, New York, 1988; BIOCOMPUTING: INFORMATICS AND GENOME PROJECTS, Smith, D.W., ed., Academic Press, New York, 1993; COMPUTER ANALYSIS OF SEQUENCE DATA, PART I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; SEQUENCE ANALYSIS IN MOLECULAR BIOLOGY, von Heijne, G., Academic Press, 1987; and SEQUENCE ANALYSIS PRIMER, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H., and Lipton, D., *SIAM J Applied Math* (1988) 48:1073). Methods

commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H., and Lipton, D., *SIAM J Applied Math* (1988) 48:1073. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCS program package (Devereux, J., *et al.*, *Nucleic Acids Research* (1984) 12(1):387), BLASTP, BLASTN, FASTA (Atschul, S.F. *et al.*, *J Molec Biol* (1990) 215:403).

As an illustration, by a polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence of SEQ ID NO: 1 is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include on average up to five point mutations per each 100 nucleotides of the reference nucleotide sequence of SEQ ID NO: 1. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

Similarly, by a polypeptide having an amino acid sequence having at least, for example, 95% "identity" to a reference amino acid sequence of SEQ ID NO:2 is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include on average up to five amino acid alterations per each 100 amino acids of the reference amino acid of SEQ ID NO: 2. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the

amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

**Polypeptides of the Invention**

In one aspect, the present invention relates to LbpB polypeptides (or LbpB proteins). The LbpB polypeptides include the polypeptides of SEQ ID NO:2, 4, 6, 8, or 10 (residues 1-18 is the natural signal peptide of each of the proteins, and residue Cys19 is the N-terminal amino acid which is lipidated in the natural mature protein); as well as polypeptides comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 8, or 10; and polypeptides comprising the amino acid sequence which have at least 65% identity to that of SEQ ID NO:2, 4, 6, 8, or 10 over its entire length, and still more preferably at least 70% identity, and still more preferably at least 80% identity, and even still more preferably at least 90% identity to SEQ ID NO: 2, 4, 6, 8, or 10. Furthermore, those with at least 95-99% are highly preferred. Also included within LbpB polypeptides are polypeptides having the amino acid sequence which have at least 65% identity to the polypeptide having the amino acid sequence of SEQ ID NO:2, 4, 6, 8, or 10 over its entire length, and still more preferably at least 70% identity, and still more preferably at least 80% identity, and still more preferably at least 90% identity to SEQ ID NO:2, 4, 6, 8, or 10. Furthermore, those with at least 95-99% are highly preferred.

The LbpB polypeptides provided in SEQ ID NO:2, 4, 6, 8, and 10 are the LbpB polypeptides from *Neisseria meningitidis* strains BNCV, M981, H44/76, M990, and 881607 repectively.

The LbpB polypeptides may be in the form of the "mature" protein or may be a part of a larger protein such as a fusion protein. It may be advantageous to include an additional amino acid sequence which contains secretory or leader sequences (such as the natural LbpB leader sequence; residues 1-18 in SEQ ID NO:2, 4, 6, 8, and 10), pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

Fragments of the LbpB polypeptides are also included in the invention. A fragment is a polypeptide having an amino acid sequence that entirely is the same as part,

but not all, of the amino acid sequence of the aforementioned LbpB polypeptides. As with LbpB polypeptides, fragments may be "free-standing," or comprised within a larger polypeptide of which they form a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments from about amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, and 101 to the end of LbpB polypeptide. In this context "about" includes the particularly recited ranges larger or smaller by several, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes.

Preferred fragments include, for example, truncation polypeptides having the amino acid sequence of LbpB polypeptides, except for deletion of a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus and/or transmembrane region or deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred are fragments characterized by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions. Other preferred fragments are biologically active fragments. Biologically active fragments are those that mediate LbpB activity, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also included are those that are antigenic or immunogenic in an animal, especially in a human.

Fragments should retain one or more of the biological activities of the LbpB polypeptide. Preferably, the polypeptide fragments should be continuous stretches (over 16 amino acids) of amino acid sequence derived from SEQ ID NO:2, 4, 6, 8, or 10 having an antigenic or immunogenic biological activity that the full length LbpB polypeptide from which it was derived also possesses.

Variants of the defined sequence and fragments also form part of the present invention. Preferred variants are those that vary from the referents by conservative amino acid substitutions -- i.e., those that substitute a residue with another of like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the

acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination.

Most preferred variants are those that vary from the referents by amino acid substitutions that are found in structurally equivalent positions (as shown by a homology alignment) in other LbpB sequences (for example homology alignment of 5 LbpB sequences shown in Figure 9). Especially preferred variants comprising the amino acid sequence which have at least 65% identity to that of the reference sequence (for instance SEQ ID NO: 2, 4, 6, 8, or10) over its entire length, and still more preferably at least 70% identity, and still more preferably at least 80% identity, and even still more preferably at least 90% identity. Furthermore, those with at least 95-99% are highly preferred. For instance, in Figure 9 if LbpB from BNCV is the reference sequence, a variant would encompass residues 300-308 being replaced with any of the residues in the equivalent positions in the LbpB sequences of strain H44/76 (residues 305-313 respectively), strain M990 (residues 307-315 respectively), strain M981 (residues 302-310 respectively), or strain 881607 (residues 303-311 respectively). The amino acid sequence NPDLAKSHA could therefore be substituted for STDVATNLA [ST (from M981 residues 302-303), D (from BNCV residue 302), V (from 881607 residue 306), A (from M990 residue 311), T (from H44/76 residue 310), NLA (from M990 residues 313-315)] and the resulting protein may be classed a variant, and a polypeptide of the invention.

Such substitutions can also include deletions, for instance if residues 357-366 of LbpB of strain M981 are deleted (as there are no equivalent amino acid positions in LbpB from strain BNCV - see Figure 9) such a protein may constitute a variant, and a polypeptide of the invention.

In addition, it is well known that the genomes of *Neisseria meningitidis* and other neisserial strains (for instance *Neisseria gonorrhoeae*) are genomically very homologous to each other. The genomes of *Neisseria meningitidis* and *Moraxella catarrhalis* (formerly called *Neisseria catarrhalis*) are also sufficiently homologous to allow gene exchange to take place. The LbpB equivalent proteins (or LbpB allelic variants) of neisserial strains and of *Moraxella catarrhalis* strains also constitute polypeptides of the invention if they satisfy the % sequence identity criteria described above. And yet in addition, such equivalent

proteins would also constitute polypeptides of the invention if they shared preferably at least 65 % sequence similarity with one of the reference sequences (SEQ ID NO: 2, 4, 6, 8, or 10) over its entire length as measured by the program BLAST (Altschul, S. F. *et al.*, (1997) Nucleic Acids Res. 25:3389-3402; Karlin, S. and Altschul, S. F. (1990) Proc. Natl. Acad. Sci. USA 87:2264-68; Karlin, S. and Altschul, S. F. (1993) Proc. Natl. Acad. Sci. USA 90:5873-7), and more preferably at least 70 % similarity, and still more preferably at least 80 % similarity, and even still more preferably at least 90%. Furthermore, those with at least 95-99% are highly preferred. Such proteins should bind human lactoferrin (by definition), and should also be able to cross-react with polyclonal sera against LbpB from meningococcal strains. The precise amino-acid sequence of such variants can be easily determined using information from the meningococcal polynucleotide and polypeptide sequences of SEQ ID NO:1-10.

The LbpB polypeptides of the invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring lipopolypeptides, recombinantly produced polypeptides or lipopolypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

**Polynucleotides of the Invention**

Another aspect of the invention relates to LbpB polynucleotides. LbpB polynucleotides include isolated polynucleotides which encode the LbpB polypeptides and fragments, and polynucleotides closely related thereto. More specifically, LbpB polynucleotide of the invention include a polynucleotide comprising the nucleotide sequence contained in SEQ ID NO:1, 3, 5, 7, or 9 encoding a LbpB polypeptide of SEQ ID NO: 2, 4, 6, 8, or 10 respectively, and polynucleotide having the particular sequence of SEQ ID NO:1, 3, 5, 7, or 9. LbpB polynucleotides further include a polynucleotide comprising a nucleotide sequence that has at least 65% identity over its entire length to a nucleotide sequence encoding the LbpB polypeptide of SEQ ID NO:2, 4, 6, 8, or 10, and a polynucleotide comprising a nucleotide sequence that is at least 65% identical to that of SEQ ID NO:1 from nucleotide 100 to nucleotide 2274, and a polynucleotide comprising a nucleotide sequence that is at least 65% identical to that of SEQ ID NO:3, 5, 7, or 9. In

this regard, polynucleotides at least 70% identical are more preferred, polynucleotides at least 80% identical are particularly preferred, and those with at least 90% are especially preferred. Furthermore, those with at least 95% are highly preferred and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred. Also included under LbpB polynucleotides are a nucleotide sequence which has sufficient identity to a nucleotide sequence contained in SEQ ID NO:1, 3, 5, 7, or 9 to hybridize under conditions useable for amplification or for use as a probe or marker. The invention also provides polynucleotides which are complementary to such LbpB polynucleotides.

The LbpB polynucleotides provided in SEQ ID NO:1, 3, 5, 7, and 9 are the LbpB polynucleotides from *Neisseria meningitidis* strains BNCV, M981, H44/76, M990, and 881607 repectively.

The nucleotide sequence encoding LbpB polypeptide of SEQ ID NO:2, 4, 6, 8, or 10 may be identical to the polypeptide encoding sequence contained in nucleotides 100 to 2274 of SEQ ID NO:1, or the polypeptide encoding sequence contained in SEQ ID NO:3, 5, 7, or 9 respectively, or it may be a sequence, which as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO:2, 4, 6, 8, or 10.

When the polynucleotides of the invention are used for the recombinant production of LbpB polypeptide, the polynucleotide may include the coding sequence for the mature polypeptide (residue 19 to the C-terminus of SEQ ID NO:2, 4, 6, 8, and 10) or a fragment thereof, by itself; the coding sequence for the mature polypeptide or fragment in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions (for instance residues 1 to 18 of SEQ ID NO:2, the natural signal sequence of LbpB). For example, a marker sequence which facilitates purification of the fused polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, *Proc Natl Acad Sci USA* (1989) 86:821-824, or is an HA tag, or is glutathione-s-transferase. Also preferred is LbpB fused to its natural signal sequence (residues 1 to 18 of SEQ ID NO:2). The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation

signals, ribosome binding sites and sequences that stabilize mRNA.

Further preferred embodiments are polynucleotides encoding LbpB polypeptide variants described earlier. Most preferably they comprise the amino acid sequence of the LbpB polypeptide of SEQ ID NO:2, 4, 6, 8, or 10 in which several, 10-25, 5-10, 1-5, 1-3, 1-2 or 1 amino acid residues are substituted, deleted or added, in any combination, and they retain at least one of the LbpB polypeptide's biological activities.

The present invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to polynucleotides which hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 80%, and preferably at least 90%, and more preferably at least 95%, yet even more preferably 97-99% identity between the sequences.

Polynucleotides of the invention, which are identical or sufficiently identical to a nucleotide sequence contained in SEQ ID NO:1, 3, 5, 7, or 9 or a fragment thereof, may be used as hybridization probes for cDNA and genomic DNA, to isolate full-length cDNAs and genomic clones encoding LbpB polypeptide and to isolate cDNA and genomic clones of other genes (including genes encoding homologs and orthologs from species other than *Neisseria meningitidis*) that have a high sequence similarity to the LbpB gene. Such hybridization techniques are known to those of skill in the art. Typically these nucleotide sequences are 80% identical, preferably 90% identical, more preferably 95% identical to that of the referent. The probes generally will comprise at least 15 nucleotides. Preferably, such probes will have at least 30 nucleotides and may have at least 50 nucleotides. Particularly preferred probes will range between 30 and 50 nucleotides.

In one embodiment, to obtain a polynucleotide encoding LbpB polypeptide, including homologs and orthologs from species other than *Neisseria meningitidis*, comprises the steps of screening an appropriate library under stingent hybridization conditions with a labeled probe having a nucleotide sequence contained in SEQ ID NO:1, 3, 5, 7, or 9 or a fragment thereof; and isolating full-length cDNA and genomic clones containing said polynucleotide sequence. Thus in another aspect, LbpB polynucleotides of the present invention further include a nucleotide sequence comprising a nucleotide sequence that hybridize under stringent condition to a nucleotide sequence having a

nucleotide sequence contained in SEQ ID NO:1, 3, 5, 7, or 9 or a fragment thereof. Also included with LbpB polypeptides are polypeptides comprising amino acid sequences encoded by nucleotide sequences obtained by the above hybridization conditions. Such hybridization techniques are well known to those of skill in the art. Stringent hybridization conditions are as defined above or, alternatively, conditions under overnight incubation at 42$^{o}$C in a solution comprising: 50% formamide, 5xSSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10 % dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65$^{o}$C.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to animal and human disease.

**Vectors, Host Cells, Expression**

The present invention also relates to vectors which comprise a polynucleotide or polynucleotides of the present invention, and host cells which are genetically engineered with vectors of the invention and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis *et al.*, *BASIC METHODS IN MOLECULAR BIOLOGY* (1986) and Sambrook *et al.*, *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as meningococci, streptococci, staphylococci, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and

*Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used. Such systems include, among others, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL* (*supra*).

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the desired polypeptide. These signals may be endogenous to the polypeptide (residues 1 to 18 of SEQ ID NO:2, 4, 6, 8, or 10) or they may be heterologous signals.

To express lipidated recombinant LbpB, preferably the endogenous signal peptide is encoded in the gene construct, and the preferred host system would be a bacterial host.

If the LbpB polypeptide is to be expressed for use in screening assays, generally, it is preferred that the polypeptide be produced at the surface of the cell. In this event, the cells may be harvested prior to use in the screening assay. If LbpB polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide; if produced intracellularly, the cells must first be lysed before the polypeptide is recovered.

LbpB polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic

interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

**Diagnostic Assays**

This invention also relates to the use of LbpB, antibodies against LbpB, and phage displaying antibodies against LbpB for use as diagnostic reagents. Detection of LbpB will provide a diagnostic tool that can add to or define a diagnosis of neisserial disease, among others.

Materials for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy.

Thus in another aspect, the present invention relates to a diagonostic kit for a disease or suspectability to a disease, particularly neisserial disease, which comprises:

(a) a LbpB polynucleotide, preferably the nucleotide sequence of SEQ ID NO: 1, 3, 5, 7, or 9, or a fragment thereof ;

(b) a nucleotide sequence complementary to that of (a);

(c) a LbpB polypeptide, preferably the polypeptide of SEQ ID NO: 2, 4, 6, 8, or 10, or a fragment thereof; or

(d) an antibody to a LbpB polypeptide, preferably to the polypeptide of SEQ ID NO: 2, 4, 6, 8, or 10 (and more preferably to residue 19 to the C-terminus of the polypeptide of SEQ ID NO: 2, 4, 6, 8, or 10).

(e) a phage displaying an antibody to a LbpB polypeptide, preferably to the polypeptide of SEQ ID NO: 2, 4, 6, 8, or 10 (and more preferably to residue 19 to the C-terminus of the polypeptide of SEQ ID NO: 2, 4, 6, 8, or 10).

It will be appreciated that in any such kit, (a), (b), (c), (d) or (e) may comprise a substantial component.

**Antibodies**

The polypeptides of the invention or their fragments or analogs thereof, or cells

expressing them can also be used as immunogens to produce antibodies immunospecific for the LbpB polypeptides. The term "immunospecific" means that the antibodies have substantially greater affinity for the polypeptides of the invention than their affinity for other related polypeptides in the prior art.

Antibodies generated against the LbpB polypeptides can be obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, preferably a nonhuman, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., *Nature* (1975) 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today* (1983) 4:72) and the EBV-hybridoma technique (Cole *et al.*, MONOCLONAL ANTIBODIES AND CANCER THERAPY, pp. 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies (U.S. Patent No. 4,946,778) can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography.

Antibodies against LbpB polypeptides may also be employed to treat neisserial disease (for example meningitis), among others. They may also be used to diagnose the disease.

**Vaccines**

Another aspect of the invention relates to a method for inducing an immunological response in a mammal (preferably a human) which comprises inoculating the mammal with LbpB polypeptide or epitope-bearing fragments, analogs, outer-membrane vesicles or cells (attenuated or otherwise), adequate to produce antibody and/or T cell immune response to protect said animal from neisserial disease, among others. Yet another aspect of the invention relates to a method of inducing immunological response in a mammal (preferably a human) which comprises, delivering LbpB polypeptide via a

vector directing expression of LbpB polynucleotide *in vivo* in order to induce such an immunological response to produce antibody to protect said animal from diseases.

A further aspect of the invention relates to an immunological composition or vaccine formulation which, when introduced into a mammalian host (preferably a human), induces an immunological response in that mammal to a LbpB polypeptide wherein the composition comprises a LbpB gene, or LbpB polypeptide or epitope-bearing fragments, analogs, outer-membrane vesicles or cells (attenuated or otherwise). The vaccine formulation may further comprise a suitable carrier. The LbpB vaccine composition is preferably administered orally or parenterally (including subcutaneous, intramuscular, intravenous, intradermal etc. injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

Yet another aspect relates to an immunological/vaccine formulation which comprises the polynucleotide of the invention. Such techniques are known in the art, see for example Wolff *et al.*, *Science*, (1990) *247*: 1465-8.

**Screening Assays**

The LbpB polypeptide of the present invention may be employed in a screening process for compounds which antagonize (antagonists, or otherwise called inhibitors) of the LbpB polypeptide of the present invention. Thus, polypeptides of the invention may also be used to identify antagonists from, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These antagonists may be natural or modified

substrates, ligands, receptors, enzymes, etc., as the case may be, of the polypeptide of the present invention; or may be structural or functional mimetics of the polypeptide of the present invention. See Coligan *et al.*, *Current Protocols in Immunology* 1(2):Chapter 5 (1991).

LbpB polypeptides are responsible for many biological functions, including many pathologies. Accordingly, it is desirous to find compounds and drugs can inhibit the function of LbpB polypeptide. In general, antagonists may be employed for a variety of therapeutic and prophylactic purposes for such conditions as neisserial disease.

In general, such screening procedures may involve using appropriate cells which express the LbpB polypeptide or respond to LbpB polypeptide of the present invention. Such cells include cells from mammals, yeast, *Drosophila* or *E. coli*. Cells which express the LbpB polypeptide (or cell membrane containing the expressed polypeptide) or respond to LbpB polypeptide are then contacted with a test compound to observe binding, or inhibition of a functional response. The ability of the cells which were contacted with the candidate compounds is compared with the same cells which were not contacted for LbpB activity.

The assays may simply test binding of a candidate compound wherein adherence to the cells bearing the LbpB polypeptide is detected by means of a label directly or indirectly associated with the candidate compound or in an assay involving competition with a labeled competitor.

Further, the assays may simply comprise the steps of mixing a candidate compound with a solution containing a LbpB polypeptide to form a mixture, measuring LbpB activity in the mixture, and comparing the LbpB activity of the mixture to a standard.

The LbpB cDNA, protein and antibodies to the protein may also be used to configure assays for detecting the effect of added compounds on the production of LbpB mRNA and protein in cells. For example, an ELISA may be constructed for measuring secreted or cell associated levels of LbpB protein using monoclonal and polyclonal antibodies by standard methods known in the art, and this can be used to discover agents which may inhibit the production of LbpB (also called antagonist) from suitably manipulated cells or tissues.

Examples of potential LbpB polypeptide antagonists include antibodies or, in some cases, oligonucleotides or proteins which are closely related to the ligands or substrates of the LbpB polypeptide, e.g., a fragment of the ligands or substrates; or small molecules which bind to the polypeptide of the present invention but do not elicit a response, so that the activity of the polypeptide is prevented.

Thus in another aspect, the present invention relates to a screening kit for identifying antagonists, ligands, or substrates for LbpB, which comprises:

(a) a LbpB polypeptide, preferably that of SEQ ID NO:2, 4, 6, 8, or 10;

(b) a recombinant cell expressing a LbpB polypeptide, preferably that of SEQ ID NO:2, 4, 6, 8, or 10;

(c) a cell membrane expressing a LbpB polypeptide; preferably that of SEQ ID NO: 2, 4, 6, 8, or 10; or

(d) antibody to a LbpB polypeptide, preferably that of SEQ ID NO: 2, 4, 6, 8, or 10.

It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component.

**Formulation and Administration**

Peptides, such as the soluble form of LbpB polypeptides, and antagonist peptides or small molecules, may be formulated in combination with a suitable pharmaceutical carrier. Such formulations comprise a therapeutically effective amount of the polypeptide or compound, and a pharmaceutically acceptable carrier or excipient. Such carriers include but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. Formulation should suit the mode of administration, and is well within the skill of the art. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention.

Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

Preferred forms of systemic administration of the pharmaceutical compositions include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for

systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if properly formulated in enteric or encapsulated formulations, oral administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels and the like.

The dosage range required depends on the choice of peptide, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are in the range of 0.1-100 μg/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

**Examples**

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples illustrate, but do not limit the invention.

Example 1: Bacterial strains and growth conditions

The bacterial strains used are listed in Table 1. Meningococci were cultured overnight on GC agar plates (Difco), supplemented with Vitox (Oxoid) in a humid 5% $CO_2$- atmosphere at 37°C. Optimal expression of iron regulated proteins was achieved by adding 5 μg/ml of the iron chelator ethylenediamine di-o-hydroxyphenylacetic acid (EDDA, Sigma). For preparation of samples for SDS-PAGE, immunoblotting, and for isolation of chromosomal DNA, cells were grown as described before (Pettersson *et al.*, 1993).

*E. coli* strain Y1090 (Young and Davis, 1983), which was used to propagate the λgt11 phage, was grown in Luria-Bertoldi (LB) medium supplemented with ampicillin (100 μl/ml), 0.2% maltose and 10 mM $MgCl_2$. Strain DH5α, used for cloning, was grown in LB medium, supplemented with 100 μl/ml ampicillin, 25 μg/ml kanamycin, or 100 μg/ml erythromycin, when needed for selection of recombinants. After transformation with pEMBL19-derivatives, cells were plated on LB plates supplemented with the appropriate antibiotic, and with 5-bromo-4-chloro-3-indoyl-β-D-galactoside (40 μg/ml), and 0.5 mM isopropyl-β-D-thiogalactopyranoside to screen for plasmids with inserts. Strain PC2494, used for preparing single stranded DNA, was kept on minimal medium plates, supplemented with 5 μg/ml thiamine and 0.2% glucose.

Example 2:

2A: Preparation of mouse antiserum against peptides

Peptides (Fig. 3) were synthesised using an automated multiple peptide synthesiser and were coupled to tetanus toxoid as described (van der Ley *et al.*, 1991). BALB/c mice were immunised with 50 μg of peptide and 20 μg of Quil A as an adjuvant. Two boosters were given. Serum was collected 54 days after the first injection.

2B: Identification of the *lbpB* gene product

To investigate whether the putative *lbpB* gene encodes a protein, antisera were raised against synthetic peptides (indicated as A1-E1 in Fig. 3) that were based on the deduced amino acid sequence of the partial open reading frame. The antisera were tested on Western blots against whole cells of strain BNCV grown under iron limitation. Sera against peptide B1 did not show any reaction (data not shown). Antisera against the other peptides reacted with a band of approximately 95 kDa (Fig. 1). Since this band was lacking in the *lbpB* mutant (constructed as described below) (Fig. 1, lanes 3 and 7), it was concluded that the *lbpB* gene is expressed in the wild-type strain, and that it encodes a protein with an apparent molecular weight (Mr) of 95,000. Some of the sera against peptides D1 and E1 showed an additional reaction with a band with an Mr of 60,000 (Fig. 1). Both of these peptides contain the sequence VVFGAK, which is also present in TbpB (Fig. 3). Therefore, the 68 K band could be TbpB. To test this possibility, a TbpB mutant, N91, and its parental strain B16B6, were tested in Western blots. Serum 19-1 (against peptide E1) reacted with two bands of 95 K and 68 K respectively, in strain B16B6, but only with the 95 K band in strain N91. This result indicates that the 68 K band is indeed TbpB (data not shown).

Example 3: SDS-PAGE and immunoblotting

SDS-PAGE of whole cell proteins was performed as described previously (Pettersson *et al.*, 1990, 1993). In experiments where denaturation of LbpB had to be prevented the following modifications were included. The sample buffer contained no β-mercaptoethanol. The outer membrane complexes were not heated at 95°C in sample buffer before electrophoresis, but incubated either on ice or at 37°C for 10 min. In the lactoferrin binding experiment, the polyacrylamide gel was composed of a 5% (w/v) stacking gel and an 8% (w/v) resolving gel containing 0.05% (w/v) SDS. The electrode buffer contained only 0.05% instead of 0.1% SDS. Electrophoresis was carried out at a constant voltage of 100 V for 2 h at 4°C. Standard sample buffer with 2% SDS was used.

Electrophoresis of outer membrane proteins to detect folded forms of LbpB was performed with the PhastSystem (Pharmacia) according to the instructions of the manufacturer, using 7.5% (w/v) homogeneous polyacrylamide gels with SDS buffer strips.

Immunoblotting was performed as described previously (Pettersson *et al.*, 1990, 1993). In the case of PhastSystem gels, the blotting buffer contained 0.05% (w/v) SDS and the activity of the peroxidase was detected with the ECL system according to the instructions of the manufacturer (Amersham). The mouse antisera were used at a dilution of 1:500. The LbpA-specific monoclonal antibodies mn98K1 and mn98K2 (Pettersson *et al.*, 1993) were used as a cocktail at a dilution of 1:2000 each.

Example 4:

4A: Cloning and sequencing strategies

The λgt11 gene library from strain BNCV was originally provided by E.C. Gotschlich (The Rockefeller University, New York, USA). The library was propagated in *E. coli* strain Y1090 and screened with DNA probes BE1 and AP6 (Fig. 2). BE1 was prepared by isolation of the 355 bp *Bst*EII-*Eco*RI-fragment of plasmid pAM1 (Pettersson *et al.*, 1993). AP6 was the 417 bp *Eco*RI-*Eco*RV-fragment prepared from plasmid pAM6. Probe labelling, plaque blotting, and detections were performed as described (Pettersson *et al.*, 1993), using the DIG DNA Labeling and Detection kit (Boehringer Mannheim). λ DNA was isolated (Sambrook *et al.*, 1989) and the inserts were subcloned in the phagemid pEMBL19. Plasmid DNA was isolated on Jetstar mini columns (Genomed) as described by the manufacturer. Single-stranded DNA was propagated using the helper phage VCSM13 (Stratagene).

Chromosomal DNA was isolated as described (Ausubel *et al.*, 1989). The DNA was digested with *Acc*I and *Dra*I, and separated on a 1% agarose gel. Southern blotting was performed as described (Pettersson *et al.*, 1993). The probe ES1 (Fig. 2) was prepared by isolation of the 320 bp *Eco*RI-*Sal*I-fragment from pAM13, and labelled as above. The probe reacted with a 1.5 kb fragment in the *AccI/Dra*I digested chromosomal DNA on a Southern blot. Fragments of 1.5 kb were isolated from gel and ligated in pEMBL19. The ligation mix was PCR amplified with the M13 universal primer (Pharmacia) and the LB11 primer (Table 2). Goldstar polymerase, a Taq polymerase derivative (Eurogentec) was used for PCR amplification according to the instructions from the manufacturer. The PCR product of 1.3 kb was purified from agarose gel.

DNA sequencing was performed manually using the deaza G/A T7 sequencing

mixes (Pharmacia) or automatically using the ABI Prism 310 Genetic Analyzer (Perkin Elmer). For automatic sequencing, the labelling was done with the Dye Terminator Cycle sequencing kit (Perkin Elmer). Internal primers (synthesised by Pharmacia or Gibco BRL) and the M13 universal and reverse primers (Pharmacia) were used for sequencing of single-stranded DNA, double-stranded plasmid DNA or the PCR product.

Similar strategies were used to sequence the *lbpB* gene from the H44/76 and M981 stains of *N. meningitidis*.

4B: Cloning and sequencing of the *lbpB* gene

To clone the missing part of the *lbpB* gene, a λgt11 gene library of strain BNCV was screened with DNA probes. Two different lambda clones were found. The inserts were subcloned in pEMBL19, resulting in plasmids pAM6 and pAM13 (Fig. 2), and sequenced. The promoter and the beginning of the *lbpB* gene were not found in this way. Several other attempts to clone the 5' end of the gene failed, suggesting that its expression is toxic to *E. coli*. To obtain the rest of the sequence, a rich bank was prepared of *Acc*I- and *Dra*I-digested chromosomal DNA. Chromosomal DNA fragments of approximately 1.5 kb were ligated in pEMBL19, and a PCR amplification was performed directly on the ligation mix, using a primer (LB11, see Fig. 2) based on the known part of the *lbpB* sequence and an M13 primer. The resulting PCR product (Fig. 2) was used directly for sequencing. This strategy avoids cloning of the possibly toxic gene in *E. coli*.

Sequencing of the various *lbpB* fragments revealed an open reading frame of 2,175 bp. It encodes a protein of 725 amino acid residues (Fig. 3) with a molecular mass of 79.4 kDa. Analysis of the N-terminal sequence revealed the characteristics of a signal sequence recognised by signal peptidase II (von Heijne, 1989). Such signal sequences are present in the precursors of lipoproteins, which are acylated at the N-terminal cysteine residue of the mature protein. A similar signal sequence was found in the TbpB protein, which was indeed proven to be lipid modified (Anderson *et al.*, 1994). The mature LbpB protein has a calculated molecular mass of 77.5 kDa, which is considerably lower than the apparent molecular mass of 95 kDa observed in sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) (Fig. 1). Screening of the Swiss Prot data base for similarities to other proteins revealed homology to TbpB of *Neisseriae* and *Actinobacillus*

*pleuropneumoniae*. The highest homology, 33 % identity (using the PALIGN program), was found to TbpB of *N. meningitidis* strain B16B6 (Legrain *et al.* 1993) (Fig. 3). In the TbpB protein, some internal repeats were found, and it has been proposed that the molecule has a bi-lobed structure that is evolved after an internal duplication (Fuller *et al.*, 1996, Renauld-Mongénie *et al.*, 1996). When the N-terminal 354 amino acids of the mature LbpB protein were aligned with the C-terminal 353 amino acids, 30 % identity and 10 % similarity was found (data not shown). This result suggests that also LbpB may exist in a bi-lobed structure. The isoelectric point of the protein is 4.5. Two long stretches, rich in acidic residues, could be discerned in the sequence (Fig. 3). Since these stretches are lacking in TbpB, they could be important for binding of lactoferrin, which is, in contrast to transferrin, a positively charged molecule.

In the promoter area, a typical Shine-Dalgarno sequence could be discerned. In addition, putative -10 and -35 boxes were found (Fig. 4). A sequence reminiscent of a Fur-binding site overlaps the -10 box. Fur acts, in conjunction with $Fe^{2+}$, as a repressor of iron-regulated genes, by binding to a 19 bp sequence in the promoter region (Bagg and Neilands, 1987). The consensus sequence of such a Fur-box is GATAATGATAATCATTATC, and 16 of the 19 bp of this sequence are conserved in this element in the *lbpB* promoter. Further upstream of the promoter, a direct repeat of 131 bp was found (data not shown). This sequence is present at least twice at this position. The same direct repeat was found downstream of the *lbpA* gene (Prinz *et al.*, unpubiished observation). A FASTA homology search revealed homology of this repeat to a number of neisserial sequences, mostly flanking open reading frames (data not shown).

The sequence homology between the LbpB proteins of the BNCV and M981 strains of *N. meningitidis*, and between the LbpB proteins of the BNCV and H44/76 strains of *N. meningitidis*, is 72.7% and 78.5% respectively.

Example 5:

5A: Construction of isogenic mutants

Plasmids pAM23 and pAM6 were used for insertional inactivation of *lbpA* and *lbpB*, respectively. The erythromycin resistance ($Erm^r$) cassette from pER2 (Jennings *et al.*, 1993) was excised with *Cla*I and *Hind*III. The fragment was treated with T4 DNA

polymerase and ligated to *Eco*RV-digested pAM23, resulting in plasmid pAM23E. The kanamycin resistance ($Km^r$) cassette from pUC4K (Pharmacia) was excised with *Hinc*II. Plasmid pAM6 was linearized with *Bgl*II and treated with T4 DNA polymerase. The $Km^r$-cassette was ligated in this site, resulting in plasmid pAM6K. A linker composed of the oligonucleotides nus1 and nus2 (Table 2), which contains the neisserial uptake sequence (GCCGTCTGAA) and *Kpn*I-compatible single-stranded ends, was cloned in the *Kpn*I-site of pAM6K, resulting in plasmid pAM6K-nus. The antibiotic resistance genes were in the same directions as *lbpA* and *lbpB* in plasmids pAM23E and pAM6K (-nus), respectively. Plasmid pAM23E, linearized with *Kpn*I, was used to transform strain H44/76 as described previously (van der Ley and Poolman, 1992). Transformants were selected on GC plates containing 5 µg of erythromycin per ml. Correct gene replacement in one of the transformants, designated CE1449, was verified by PCR, using primers FW5 and DVAS2 (Table 2), and by Southern blot analysis using probe AP23 (Fig. 2; isolated as the 184 bp *Ssp*I-*Hind*III-fragment from pAM23). For Southern blot, chromosomal DNA was digested with *Cla*I and *Sal*I. The isogenic mutants in strain BNCV were prepared by electroporation (Genco *et al.*, 1991) since this strain appeared not to be transformable. Chromosomal DNA from *lbpA* mutant CE1449 was used to make the *lbpA* mutant. Transformants were selected on GC plates with erythromycin and verified as mentioned above. Plasmid pAM6K-nus was used to make the *lbpB* mutant. Transformants were selected on GC plates containing 100 µg/ml of kanamycin. Correct gene replacement was verified by PCR, using primers SDA1 and PR1 (Table 2), and by Southern blot using probe AP23.

5B: Construction of isogenic mutants

To verify the identity of the 94 kDa protein and to investigate the role of the individual lactoferrin-binding proteins in lactoferrin binding and utilisation, a set of isogenic derivatives of BNCV lacking either LbpA or LbpB was constructed as described in Example 5A. Correct gene replacements were verified in PCR reactions and by Southern blotting (data not shown). Expression of LbpA and LbpB in the mutants was checked on Western blots (Fig. 5). The *lbpA* mutant CE1452 did not express LbpA (Fig. 5A, lane 4), and the *lbpB* mutant CE1454 did not express the 94 kDa protein (Fig. 5B, lane 6). This

result confirms that the *lbpB* gene is indeed expressed in the wild-type strain, and that it encodes a protein with an $M_r$ of 94,000, which is considerably higher than its calculated molecular mass of 77.5 kDa. Furthermore, the results from Fig. 4 show that the inactivation of *lbpB* does not have a polar effect on LbpA expression (Fig. 5A, lane 6). This was anticipated, since the kanamycin resistance cassette that was inserted in *lbpB* does not contain a transcriptional terminator. The previously described spontaneous *lbpA* mutant CE 1402 (Pettersson *et al*, 1994b) appeared to lack LbpB expression as well (Fig. 5B, lane 8). Since both this mutant and BNCV are derivatives of strain M986, its genetic background is the same as that of the other mutants. Expression of both LbpA and LbpB appeared to be iron-regulated (Fig. 5). A weak expression of LbpA was seen in strain CE1454 even when the cells were grown without an iron chelator (Fig. 5A, lane 5). This expression is probably due to transcription from the promoter of the kanamycin resistance gene in *lbpB*. However, also in this case, the expression of the LbpA was increased severalfold when the strain was grown in the presence of an iron chelator (Fig. 5A, lane 6).

Example 6:

6A: Lactoferrin binding assay

Lactoferrin binding to whole cells was assessed in an ELISA-type assay. Recombinant human lactoferrin, produced in *Aspergillus avamori*, was kindly provided by Agennix Inc., Houston, Texas, USA. The lactoferrin was saturated with iron as described (van Berkel *et al.*, 1995), with the following modifications. $FeCl_3$ was used instead of $Fe(NO_3)_3$, and the dialysis was done against 5 mM $Na_2HPO_4/NaH_2PO_4$-buffer, pH 7.7 for 4 h. Colonies from plates were suspended in Tris-buffered saline, pH 7.5 (TBS) and killed by heating for 30 min at 56°C. Samples (100 µl) with an optical density at 620 nm of 0.05 were dispensed into the wells of a microtiter plate. The samples were allowed to dry overnight at 37°C. The assay was carried out at 37°C. Nonspecific binding was prohibited with 100 µl of blocking solution containing 0.5% Protifar (Nutricia) and 0.1% Tween 20 in TBS for 1 h. After blocking, the wells were filled with various concentrations of lactoferrin in blocking solution. The concentration of lactoferrin in the wells varied from 3.125 to 200 ng/ml. After incubation for 1 h, and three washes with tap water, a peroxidase-coupled rabbit polyclonal antiserum against

human lactoferrin (ICN Biomedicals) was added to the wells. The antibody was used at a dilution of 1:5000 in blocking buffer. After incubation for 1 h and three washes with tap water, the amount of peroxidase was detected (Abdillahi and Poolman, 1987).

Lactoferrin binding on a blot was performed as follows. Unspecific binding was blocked by incubating the membrane in 0.2 M $Na_2HPO_4/NaH_2PO_4$ buffer, pH 5.7 containing 0.1% Tween-20 and 0.5% Protifar (Nutricia) for 2 h. The blot was incubated with 1.2 $\mu gml^{-1}$ peroxidase-conjugated human lactoferrin (Pettersson *et al.*, 1993) in blocking buffer for 1 h and washed three times with blocking buffer. The activity of the peroxidase was detected with the ECL system according to the instructions of the manufacturer (Amersham).

6B: Plate feeding assays

Meningococci were grown overnight in TSB supplemented with Vitox, as described in Example 1. Of the overnight culture, 300 µl were suspended in 3 ml of top agar (1% GC agar with 20 µg EDDA per ml, cooled down to 42°C) and immediately plated on GC agar plates supplemented with Vitox and 20 µg EDDA per ml. Drops (10 µl) of recombinant human lactoferrin (11% iron saturated, or saturated as described above) were spotted on the plates. The concentration of lactoferrin in the drops was 10 and 20 mg/ml, respectively. Plates were grown overnight.

6C: Binding of lactoferrin to folded LbpB on blots

To investigate whether lactoferrin can bind to LbpB on blots, SDS-PAGE conditions were sought under which the LbpB is not denatured (see Example 6A). When samples were not heated in sample buffer prior to electrophoresis, a faster migrating form of the LbpB protein could be detected, probably representing the native, folded form of the protein (Fig.6A). This form had an Mr of approximately 80 kDa. After heating for 10 min at 95°C, the LbpB protein was fully denatured and migrated at the 94 kDa position (Fig. 6A, lane 2). Interestingly, only the serum against the peptide A1 (Fig. 2) reacted with the faster migrating form of the protein. This peptide contains one of the two stretches, rich in negatively charged amino acids and possibly implicated in lactoferrin binding. The binding of the antibodies to the folded protein suggests that this part of the

protein is exposed, whereas all other peptide epitodes are hidden in the folded structure of LbpB.

Binding of lactoferrin to the folded LbpB protein was subsequently assessed. Outer membrane proteins of strain BNCV were blotted to a nitrocellulose membrane, and were incubated with peroxidase-coupled human lactoferrin. The specificity of the lactoferrin binding appeared to be extremely sensitive to the incubation conditions, most importantly, the pH. Under optimized conditions, lactoferrin bound specifically to a protein band with a Mr of 80 kDa (Fig. 6B, lanes 1 and 2). No binding was observed when the samples were heated for 10 min at 95°C prior to SDS-PAGE (Fig. 6B, lane 3). The band was not detected in the samples of the *lbpB* mutant CE1454 (data not shown). Hence, it is concluded that the faster migrating form of the LbpB protein, probably representing the folded form of the protein, is capable of lactoferrin binding.

6D: Lactoferrin binding and utilisation in whole cells

Lactoferrin binding to whole cells was investigated in an ELISA-type assay. The ELISA plates were coated with whole cells of strain BNCV of the isogenic mutants, and lactoferrin, in various concentrations, was added to the wells. Binding of lactoferrin to the cells was probed with a peroxidase-conjugated antibody against human lactoferrin (Fig. 7). The *lbpB* mutant was slightly reduced in its ability to bind lactoferrin. The *lbpA* mutant bound lactoferrin less effectively that the *lbpB* mutant, whereas the double mutant bound virtually no lactoferrin at all (Fig. 7).

The ability to use lactoferrin as a sole source of iron was investigated in plate feeding assays. Meningococci were grown under iron limitation on plates, drops of recombinant human lactoferrin were spotted on the plates, and growth stimulation was monitored. The *lbpB* mutant was able to grow on lactoferrin, whereas the *lbpA* mutant was not (Fig. 8). The lactoferrin was 11 % iron saturated. The same experiment was performed with iron-loaded lactoferrin with essentially the same results (data not shown). These data demonstrate that the LbpA protein is necessary for iron uptake via lactoferrin, whereas LbpB does not seem to be essential.

Example 7:

To study the variability of the meningococcal LbpB protein, the *lbpB* genes from four further strains were sequenced: H44/76, M990, M981, 881607 (see Table 1).

7A: Sequencing of *lbpB* from four further meningococcal strains - Methods

Bacteria were cultured in the same way as described in Example 1. Chromosomal DNA was isolated from bacteria grown on plates. After overnight growth, bacteria were scraped from the plates and suspended in 1.5 ml 10 mM Tris-HCl, 10 mM EDTA, pH 8 and 10 μl of lysozyme (10 mg/ml) was added. The suspension was incubated for 15 min at room temperature, before 1.5 ml of 2 % Triton X-100, 50 mM Tris-HCl, 10 mM EDTA, pH 8 was added. After 15 min incubation, 10 μl of proteinase K (10 mg/ml) was added. The tubes were incubated for 30 min at room temperature. The mixture was extracted once with phenol, chloroform, isoamylalcohol (mixed in ratio 24:24:1), and once with chloroform saturated with water. The chromosomal DNA was precipitated with ethanol.

Chromosomal DNA was used to PCR amplify the *lbpB* genes. Primers LB20 and REV2 (Table 3) were used on strains H44/76, M990, and 881607. Primers LB20 and LB23 were used on strain M981. The primers are based on the sequence of *lbpB* from strain BNCV. LB20 binds upstream of the *lbpB* gene, and LB23 and REV2 at the beginning of the *lbpA* gene. LB23 has an extra *Bam*HI site at the 5' end. Goldstar polymerase, a *Taq* polymerase derivative (Eurogentec) was used for PCR amplifications according to the instructions from the manufacturer. The annealing temperature was in all cases 50°C, and 30 cycles were performed. PCR products were purified from agarose gels, using β-agarase (New England Biolabs) according to the instructions of the manufacturer.

DNA sequencing was performed by gene walking using primers designed for the *lbpB* genes. Primers were synthesized by Gibco BRL. Sequencing was done automatically using the ABI Prism 310 Genetic Analyser (Perkin-Elmer). The labelling was done with the Dye Terminator Cycle Sequencing Kit (Perkin-Elmer).

The computer programs TRANSL, PALIGN and CLUSTAL from the software package PC Gene 6.70 (IntelliGenetics) were used to translate the nucleotide sequence

into amino acid sequence, for pairwise alignment of sequences and for multiple alignment, respectively.

7B: Sequencing of *lbpB* from four further meningococcal strains - Results

The nucleotide sequences of the *lbpB* genes of the four strains are shown in SEQ ID NO:3, 5, 7, and 9. The nucleotide sequences were translated into amino acid sequences and an alignment of the five known sequences of the LbpB proteins is presented in Fig. 9. On the amino acid level, the identity between the LbpB proteins of the different strains was 70-80%. A pairwise comparison of identities is summarized in Table 4.

Example 8:

The expression level of LbpB in *Neisseria meningitidis* is very low; the protein could not be detected when outer membrane protein patterns were analysed by SDS-PAGE. For immunological and structural/functional studies of the LbpB protein, a construct was made for expression of the protein in *Escherichia coli*. To facilitate purification of the recombinant protein, the protein encoded by the construct contained a His-tag , and lipid modification of the N terminus was prevented by replacement of the native signal sequence and first two amino acid residues of the mature domain.

8A: Expression of recombinant LbpB - Bacterial strains and growth conditions

The meningococcal strain BNCV (-:2a:P1.2) was cultured overnight on GC agar plates (Difco), supplemented with Vitox (Oxoid) in a humid 5% $CO_2$ atmosphere at 37°C. The construct encoding the recombinant LbpB protein was expressed in the *E. coli* strain CE1448 (generously provided by C. Jansen), which is a *htrA ompT* derivative of strain CE1224 (Tommassen *et al.*, 1983). The strain was grown at 37°C in a Hepes-buffered synthetic medium (Tommassen and Lugtenberg, 1980) supplemented with growth requirements due to auxotrophic mutations and with 1.32 mM $K_2HPO_4$ (phosphate-replete conditions). After overnight growth, the culture was diluted 1:13.5 into the same medium, but without $K_2HPO_4$ (phosphate-depleted conditions) and grown for 6 h at 37°C.

8B: Expression of recombinant LbpB - Cloning in *E. coli*

Chromosomal DNA was isolated from meningococcal cells grown overnight on plates. Bacteria were scraped from the plates, suspended in 1.5 ml 10 mM Tris-HCl, 10 mM EDTA, pH 8, and 10 μl of lysozyme (10 mg/ml) was added. The suspension was incubated for 15 min at room temperature, before 1.5 ml of 2 % Triton X-100, 50 mM Tris-HCl, 10 mM EDTA, pH 8 was added. After 15 min incubation, 10 μl of proteinase K (10 mg/ml) was added. The tubes were incubated for 30 min at room temperature. The DNA was extracted from the mixture by adding phenol/ chloroform/ isoamylalcohol (24:24:1 by volume), and further purified by extraction with chloroform saturated with water. The chromosomal DNA was precipitated with ethanol.

The chromosomal DNA was used as a template to amplify the part of the *lbpB* gene corresponding to the mature LbpB by PCR using primers LB22 and LB23 (Table 5). LB22 primes at a site corresponding to the N-terminal part of LbpB and introduces a *Pst*I-site in the PCR product. LB23 primes just downstream of *lbpB* at the beginning of the *lbpA* gene and introduces a *Bam*HI- site. *Pwo* polymerase (Boehringer Mannheim), a proof-reading enzyme, was used in the PCR reaction, according to the instructions provided by the manufacturer. The annealing temperature was 60°C, and 30 cycles were performed. The PCR product was isolated from a gel, using β-agarase (New England Biolabs) according to the manufacturer's instructions. The PCR product was digested with *Pst*I and *Bam*HI and ligated into pJP29 (Fig. 10A), which had also been digested with *Pst*I and *Bgl*II. In the resulting construct, pAM31, the *Bam*HI and *Bgl*II sites are lost. The LbpB protein is expressed in this construct from the *phoE* promoter and contains the PhoE signal sequence instead of the authentic signal sequence. Furthermore, the first two residues from the N-terminus were changed from Cys and Ile to Ala and Val, respectively. To facilitate the purification of the protein, a His-tag was inserted between the signal sequence and the mature part of LbpB. pAM31 was digested with *Pst*I and ligated to a linker composed of the oligonucleotides VGO12a and VGO13a (Table 5), resulting in plasmid pAM32 (Fig. 10A). The *Pst*I-site is lost after ligation. The linker codes for six His residues and a factor Xa cleavage site (Fig. 10B).

8C: Purification of recombinant LbpB

The recombinant LbpB was produced in strain CE1448 containing pAM32. Phosphate-limited cells from a 5 litre culture were harvested after 6 h of growth. Cells were washed once with 500 ml physiological salt solution and resuspended in 150 ml of 10 mM Tris-HCl, 5 mM EDTA, pH 8. The suspension was frozen at -20°C overnight. The cells were thawed and three protease inhibitor cocktail tablets (Complete™, Boehringer Mannheim) were added. The cells were pressed twice through a French press at a pressure of 8000 psi. Unbroken cells were removed by centrifugation in a Sorvall GSA rotor at 5000 rpm for 20 min. The supernatant was centrifuged in a Beckman Ti60 rotor at 40,000 rpm for 90 min. The cell envelopes were dissolved in 5 mM $Na_2HPO_4$-$NaH_2PO_4$- buffer, pH 7.6.

The cell envelopes were extracted twice with 2% n-octyl-oligo-oxyethylene (Octyl-POE) at 37°C. The first extraction was done for 1 h, and the second for 3 h. In between and after the extractions, non-soluble proteins were pelleted by centrifugation in a Beckman TLA100.2 rotor at 100,000 rpm for 1 h. Supernatants, containing the LbpB protein, were combined and added to Ni-NTA agarose. Purification of the protein was done in batch under native conditions, according to the instructions provided by the manufacturer (Qiagen). The concentrations of imidazole and NaCl were 20 mM and 300 mM, respectively, during binding and washing. In total, 2 ml of Ni-NTA agarose was used, divided over 10 tubes. Elution was performed in steps with 3 ml of 100 mM, 200 mM, and 250 mM imidazole, respectively. After elution, the protein was dialyzed twice in a Spectra/Por 2 dialysis bag (Spectrum) against 2.5 l of phosphate-buffered saline. The protein was concentrated in a Fugisept Maxi Centrifugal Concentrator (Intersept) with a cut-off of 10 kDa. The centrifugation was performed in a Sorvall GSA rotor at 5000 rpm, until the total volume was 1-1.5 ml.

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Lugtenberg *et al.* (1975) with a few modifications. The polyacrylamide gel was composed of a 5% stacking gel and an 11% resolving gel, containing no SDS. When denaturation of LbpB had to be prevented, the sample buffer (Lugtenberg *et al.*, 1975) contained no β-mercaptoethanol, and the samples were kept at 0°C before PAGE. To denature LbpB, the sample buffer was supplemented with β-mercaptoethanol, and the samples were boiled

for 5 min. Electrophoresis was carried out at a constant current of 20 mA at 4°C. The gel was stained with Coomassie Brilliant Blue.

8D: Expression and purification of recombinant LbpB - Results

A recombinant form of LbpB of *N. meningitidis* strain BNCV was expressed in the *E. coli* strain CE1448. A construct, pAM32, was made encoding a recombinant protein consisting of the signal sequence of PhoE, a His-tag and the mature LbpB protein (Fig. 10A). The protein is expressed from the *phoE* promoter under phosphate limitation. The authentic type II signal sequence of LbpB is replaced by a type I signal sequence, and a His-tag followed by a Factor Xa cleavage site is inserted between the signal sequence and the mature LbpB. Furthermore, the first two amino acids of the mature LbpB, Cys and Ile were changed into Ala and Val (Fig. 10B). Consequently, the recombinant protein cannot be lipid-modified at an N-terminal Cys. The recombinant LbpB protein fractionated with the membranes, and not with the soluble proteins (data not shown). Therefore, it had to be extracted from the membrane with a detergent. Octyl-POE was used because it solubilized about 50% of the total amount of recombinant LbpB from the membrane. Furthermore, when the extracted protein is not denatured by boiling in sample buffer, it migrates faster in PAGE than the denatured protein (data not shown), suggesting that the protein was correctly folded. After extraction, the His-tagged protein was purified by Ni- affinity chromatography. Most of the protein eluted in the 100 mM and 200 mM imidazole fractions. However, all fractions were combined before dialysis. The protein was pure as evaluated on a Coomassie Brilliant Blue-stained gel (Fig. 11), and most of it was present in the folded form, which migrates faster during PAGE than the denatured form. The folded form of LbpB, but not the denatured form, was shown in Example 6 to bind lactoferrin on a blot.

Example 9:

To study the immunogenicity of the LbpB protein in man, the presence of antibodies recognizing the LbpB protein of strain BNCV in human convalescent sera was tested in immunoblots.

9A: Immunogenicity of LbpB in man - Methods

Ten human convalescent sera were obtained from SmithKline Beecham Biologicals SA, Belgium, and seven sera from the National Institute of Public Health and the Environment, The Netherlands (Table 6). The individuals had been infected with strains of various sero- and subtypes

Recombinant LbpB was isolated using the procedure described in Example 8. Polyacrylamide gel electrophoresis (PAGE) was performed as described by Lugtenberg *et al.* (1975) with a few modifications. The polyacrylamide gel was composed of a 5% stacking gel and an 11% resolving gel, containing no SDS. When denaturation of LbpB had to be prevented, the sample buffer (Lugtenberg *et al.*, 1975) contained no β-mercaptoethanol, and the samples were kept at 0°C before electrophoresis. To denature LbpB, the sample buffer was supplemented with β-mercaptoethanol, and the samples were boiled for 5 min. Electrophoresis was carried out at a constant current of 20 mA at 4°C.

Immunoblotting was performed as described by Pettersson *et al.* (1993). Human sera were diluted 1:500. Peroxidase-conjugated rabbit anti-human IgG (Dako A/S) was used as the secondary antibody at a working dilution of 1:5000. The activity of the peroxidase was detected with the ECL system according to the instructions provided by the manufacturer (Amersham).

9B: Immunogenicity of LbpB in man - Results

The presence of LbpB specific antibodies in human sera was tested against purified, recombinant LbpB protein of strain BNCV (-:2a:P1.2) in immunoblots. Reactivity was tested both against the folded LbpB and against the denatured protein (see Fig. 12 for examples). The results are summarized in Table 6. Four of the sera reacted strongly with both the denatured and folded form of LbpB. Five sera reacted weakly with both forms, two sera weakly with only the folded form, two sera weakly with only the denatured form, and four sera did not react with LbpB at all. These results demonstrate that the meningococcal LbpB is immunogenic in man and suggest a considerable degree of immunological cross-reactivity between LbpB proteins from various strains.

Example 10: ELISA & Bactericidal tests using sera obtained from mice immunised with meningococcal cells or LbpB

10A: Immunization protocol

Immunization with *N. meningitidis* strain BNCV: Groups of 10 mice (6 weeks old Balb/C) were immunized (100 µl intraperitoneal or 100 µl subcutaneous) three times with $5 \times 10^8$ CFU of heat inactivated BNCV whole cells in SBAS2 adjuvant. The three immunizations were carried out 21 days apart, and blood was drawn on day 56 by intra cardiac punction. Sera were pooled by group.

Immunization with LbpB from *N. meningitidis* strain BNCV: This was done by the same method as above except that the 2 first immunizations were done with 10 µg of crude LbpB (*E. coli* cell envelope containing the recombinant LbpB) and the third immunization was carried out with 2.5 µg of pure LbpB (prepared in the same way as described in Example 8).

10B: Measurement of the response in Whole Cell ELISA (WCE) and purified LbpB ELISA

Flat-bottomed, 96-well Nunc immuno plates were used. 100 µl of a heat inactivated *Neisseria meningitidis* B strain [that had been grown under conditions of iron depletion conditions (fe-) using EDDA as described in Example 1] (20 µg/ml total protein) suspension in PBS was aliquotted into individual wells of plates and allowed to evaporate overnight at 37°C.

The coated plates were washed four times with 0.9% NaCl, 0.05% Tween 20 and were saturated with PBS Casein 0.3% (Merck) for 30 minutes at room temperature with stirring, and washed in the same way. 100 µl of pooled sera were 100 fold diluted in PBS Tween 20 0.05% casein 0.1%, added to the first well, then 2 fold diluted up to 12 dilutions and plates were then incubated for 30 minutes at 37°C with stirring. After washing, 100 µl of a 2000 fold dilution of Rabbit anti-mouse Immuno-globulins biotin (Dakopatts E0413) in PBS tween 20 0.05% Casein 0.3% were added and the plates were incubated in the same way as before. Plates were washed and then 100 µl of a 4000 fold dilution in PBS tween 20 0.05% of Streptavidin-biotinylated horseradish peroxidase complex were added and the plates were incubated in the same way. After washing, 100

µl of a freshly prepared solution of 4 mg O-phenyldiamine (OPDA) stain (sigma P8787) in 0.1 M citrate buffer pH 4.5 were added and plates were incubated for 15 minutes at room temperature in a dark room. The reaction was stopped by adding 50 µl 1N HCl. The absorbances were read at 490 nm.

Anti-LbpB ELISA works in the same way as WCE except the coating is different. The wells were coated with 100 µl of a solution of 0.5 µg/ml pure LbpB in 0.05 M carbonate/bicarbonate buffer pH 9.6 and incubated overnight at 37°C (not evaporated).

10C: Bactericidal assay

A culture of group B meningococci (strain H44/76) [grown either under conditions of iron depletion (fe-) as described in Example 1, or under iron rich (fe+) conditions by omitting the addition of EDDA] in the log phase of growth (OD~0.3) was suspended in sterile Hanks medium with 0.3 % BSA in order to obtain a working cell suspension adjusted to 20000 CFU/ml.

A primary reaction mixture (75 µl) was made containing 50µl/well of two-fold dilutions of test serum (Example 10A) samples (that had been heat-inactivated at 56°C for 30 min) and 25µl/well of the 20000 CFU/ml log phase group B meningococci. The reaction vials were incubated at 37°C for 15 minutes and shaked at 210 rpm. The final reaction mixture (100µl) additionally contained 25 % pretested baby rabbit serum as a complement source, and was incubated under the same conditions for 60 min. A sterile polystyrene U-bottom 96-well microtiter plate was used for this assay.

A 10 µl aliquot was taken from each well using a multichannel pipette, and was dropped onto Mueller-Hinton agar plates containing 1 % Isovitalex and 1 % heat-inactivated Horse Serum and incubated for 18 hours at 37°C in 5 % $CO_2$. Individual colonies could be counted up to 80 CFU per aliquot.

The following three test samples were used as controls: buffer + bacteria + complement; buffer + bacteria + inactivated complement; serum + bacteria + inactivated complement.

Titers were calculated using a procedure with the program Excel (Microsoft). This procedure gives a precise measurement of the dilution which corresponds to 50 % of cell killing by a regression calculation.

Example 10D: Results

Table 7 and Fig. 13 show that immunization with LbpB induces a good response against LbpB (Fig. 13A), as well as against whole cell samples from strain BNCV (the source of the recombinant LbpB) AND strain H44/76 (the LbpB of which having only 78.5% sequence identity with that of BNCV) (Fig. 13B and C). Sera obtained using an immunization schedule involving only recombinant LbpB gave a similar result (data not shown). Clearly, anti-whole cell immunization with strain BNCV leads to higher anti-whole cell ELISA for both BNCV cells and H44/76 cells (Fig. 13B and C, respectively).

In addition, immunization with recombinant LbpB from *N. meningitidis* strain BNCV induces antibodies that bind to a protein of similar molecular weight in whole cell samples from *Moraxella catarrhalis* run on an immunoblot carried out substantially as described in Example 9 (data not shown).

Table 8 and Fig. 14 show that the antibodies produced in the sera after immunization with recombinant LbpB (from strain BNCV) are bactericidal against a heterologous strain (H44/76), the LbpB of which having only 78.5% sequence identity with that of BNCV. This was also the case using sera obtained after an immunization schedule involving only recombinant LbpB (data not shown). This is true when the H44/76 has been grown in conditions containing iron (Fig. 14A) and depleted in iron (Fig. 14B). There seems to be a greater effect in conditions of iron depletion as might be expected if LbpB is expressed in greater amounts when the bacterium is under these conditions.

LbpB is therefore an immunoprotective antigen, and, furthermore, it shows evidence of providing cross-immunoprotection against heterologous strains of *N. meningitidis*.

Table 1. Bacterial strains and plasmids used.

| Strain | Description[a] | Reference/Source |
|---|---|---|
| *N. meningitidis* | | |
| H44/76 | B:15:P1.7,16 | E. Holten |
| CE1449 | H44/76 *lbpA*::Erm$^r$ | This application |
| BNCV | -:2a:P1.2 Nonencapsulated derivative of M986 | E.C. Gotschlich |
| CE1452 | BNCV *lbpA*::Erm$^r$ | This application |
| CE1452 | BNCV *lbpB*::Km$^r$ | This application |
| CE1402 | M986 *lbpA*, *lbpB* | Pettersson *et al.* 1994a |
| M990 | B:6:P1.6 | |
| 881607 | B:nt:P1.12 | |
| B16B6 | B:2a:P1.2 | A. Schryvers |
| N91 | B16B6 *tbpB* | A. Schryvers |
| M981 | B:4:nt | |
| *E. coli* | | |
| DH5α | | Laboratory stock |
| Y1090 | Amp$^r$ | Young and Davis, 1983 |
| PC2494 | *hsdR* derivative of JM101 | Phabagen Collection |
| *Plasmids* | | |
| pEMBL19 | Amp$^r$ | Laboratory stock |
| pUC4K | Km$^r$-box, Amp$^r$ | Pharmacia Biotech |
| pER2 | Erm$^r$-box in pBluescript, Amp$^r$ | Jennings *et al.*, 1993 |
| pAM6 | pEMBL19 carrying parts of *lbpA* and *lbpB* | This application |
| pAM6K | pAM6 with a Km$^r$-box from pUC4K inserted in the *Bgl*II site of *lbpB* | This application |
| pAM6K-nus | pAM6K with a neisserial uptake sequence inserted in the *KpnI*-site of the multiple cloning site of the vector | This application |
| pAM13 | pEMBL19 carrying parts of *lbpA* and *lbpB* | This application |
| pAM1 | pUC19 carrying parts of *lbpA* and *lbpB* | Pettersson *et al.* 1993 |
| pAM23 | pUC19 carrying *lbpA* and part of *lbpB* | Pettersson *et al.* 1994b |
| pAM23E | pAM23 with an Erm$^r$ - box inserted in the *Eco*RV-site of *lbpA* | This application |

[a] Serogroup, serotype and subtype are mentioned. nt: non typable.

Table 2. Primers used for PCR or linker cloning

| Name | Sequence | Remarks |
|---|---|---|
| DVAS2 | AGACCGACCCTTCGACGACTTCGG | |
| FW5 | GAAGAAGAAGCGATGGTGCGG | |
| SDA1 | CCTCTTTAGTATCTTTCTTCGCAC | |
| LB11 | CTTAATTTCATCTTTTCCC | |
| PR1 | GAGCGAGTCCGCGTTAGTGCT | Binds in $Km^r$ - cassette |
| nus1 | TTCAGACGGCTGTAC | Neisserial uptake sequence |
| nus2 | AGCCGTCTGAAGTAC | complementary to nus1 |

Table 3. Primers used to PCR amplify the four further *lbpB* genes

| Name | Sequence |
|---|---|
| LB20 | GGAGGAAAAGTAGGGATG |
| LB23 | CGGGATCCAGCCAAGGCAGTCAGGGTAAGC |
| REV2 | GCACGGACGGTAACCTCTTTCAGG |

Table 4. Pairwise identities of the LbpB sequences, in %

| | H44/76 | M990 | M981 | 881607 |
|---|---|---|---|---|
| BNCV | 78.5 | 73.8 | 72.7 | 71.4 |
| H44/76 | | 72.5 | 74.1 | 78.5 |
| M990 | | | 70.5 | 71.3 |
| M981 | | | | 80.6 |

Table 5. Primers used to express recombinant LbpB

| Name | Sequence |
|---|---|
| LB22 | AACTGCAGTCGGCGGCAATTTCGGCGTGCA |
| LB23 | CGGGATCCAGCCAAGGCAGTCAGGGTAAGC |
| VGO12a | CACCACCACCACCACCACGTGATCGAGGGGCGTGCA |
| VGO13a | CGCCCCTCGATCACGTGGTGGTGGTGGTGGTGTGCA |

Table 6. Results of the immunoblot of human sera against purified LbpB.

| Serum | Characteristics[a] | Native[b] | Denatured[b] | Source[c] |
|---|---|---|---|---|
| 262439 | B:NT:P1.4 | + | + | SKB |
| 262532 | B:15:P1.7,16 | + | + | SKB |
| 262658 | B:NT:P1.15 | - | - | SKB |
| 262716 | B:15:P1.7,16 | - | + | SKB |
| 262892 | B:2b:P1.10 | ++ | ++ | SKB |
| 262917 | B:4:NT | ++ | ++ | SKB |
| 262941 | B:1:P1.15 | + | + | SKB |
| 262987 | B:2a:P1.15 | + | + | SKB |
| 263017 | B:4:NT | - | - | SKB |
| 263021 | B:4:P1.4 | - | - | SKB |
| 69 | B:15:P1.16 | + | - | RIVM |
| 322 | B:15:P1.5 | + | - | RIVM |
| 329 | B:1:P1.4 | - | - | RIVM |
| 330 | B:1:P1.4 | ++ | ++ | RIVM |
| 187 | - | - | + | RIVM |
| 195 | - | ++ | ++ | RIVM |
| 118 | - | + | + | RIVM |

[a] Sero- and subtypes of the strain with which the patient was infected are indicated, when known. NT: not typable.

[b] ++ indicates strong reaction, + weak reaction and – no reaction with the native or denatured form of the protein.

[c] SKB: SmithKline Beecham Biologicals, RIVM: National Institute of Public Health and the Environment.

Table 7. Results of the anti Whole Cell and anti-LbpB ELISA performed as described in Example 10

| anti-LbpB response | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100[a] | 200 | 400 | 800 | 1600 | 3200 | 6400 | 12800 | 25600 | 51200 | 102400 | 204800 |
| BNCV | 0.341[b] | 0.196 | 0.107 | 0.063 | 0.033 | 0.018 | 0.014 | 0.011 | 0.013 | 0.012 | 0.009 | 0.012 |
| LbpB | 2.772 | 2.918 | 2.794 | 2.867 | 2.687 | 2.487 | 2.046 | 1.504 | 1.043 | 0.668 | 0.405 | 0.202 |
| PBS | 0.14 | 0.079 | 0.044 | 0.028 | 0.016 | 0.018 | 0.012 | 0.01 | 0.01 | 0.008 | 0.012 | 0.01 |
| anti BNCV(Fe-) response | | | | | | | | | | | | |
| | 100 | 200 | 400 | 800 | 1600 | 3200 | 6400 | 12800 | 25600 | 51200 | 102400 | 204800 |
| BNCV | 2.476 | 3.09 | 3.04 | 3.154 | 3.034 | 3.112 | 3.111 | 2.905 | 2.745 | 2.436 | 1.659 | 1.056 |
| LbpB | 1.783 | 1.856 | 1.292 | 0.914 | 0.622 | 0.385 | 0.257 | 0.185 | 0.122 | 0.106 | 0.096 | 0.089 |
| PBS | 0.687 | 0.55 | 0.358 | 0.243 | 0.154 | 0.123 | 0.099 | 0.088 | 0.083 | 0.081 | 0.031 | 0.081 |
| anti H44/76(Fe-) response | | | | | | | | | | | | |
| | 100 | 200 | 400 | 800 | 1600 | 3200 | 6400 | 12800 | 25600 | 51200 | 102400 | 204800 |
| BNCV | 2.814 | 3.003 | 2.966 | 2.976 | 2.873 | 2.66 | 2.371 | 1.862 | 1.312 | 0.873 | 0.591 | 0.452 |
| LbpB | 2.653 | 2.287 | 1.683 | 1.123 | 0.748 | 0.536 | 0.409 | 0.35 | 0.309 | 0.298 | 0.289 | 0.295 |
| PBS | 1.646 | 1.049 | 0.695 | 0.47 | 0.338 | 0.271 | 0.238 | 0.226 | 0.226 | 0.251 | 0.284 | 0.285 |

[a] Sera dilution. [b] Optical density at 490 nm.

Table 8. Results of the bactericidal activities of anti Whole Cell and anti-LbpB sera performed as described in Example 10

| Bactericidal titer against H44/76(Fe-) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 200.0[a] | 400.0 | 800.0 | 1600.0 | 3200.0 | 6400.0 | 12800.0 | 25600.0 |
| H44/76 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 97.2 |
| BNCV | 100.0 | 94.4 | 93.0 | 83.2 | 81.8 | 63.7 | 48.3 | 34.4 |
| LbpB | 10.6 | -0.5 | -0.5 | -0.5 | -0.5 | -0.5 | -0.5 | -0.5 |
| PBS | -0.5 | -0.5 | -0.5 | -0.5 | -0.5 | -0.5 | -0.5 | -0.5 |
| Bactericidal titer against H44/76(Fe+) | | | | | | | | |
| | 200.0 | 400.0 | 800.0 | 1600.0 | 3200.0 | 6400.0 | 12800.0 | 25600.0 |
| H44/76 | 100 | 98 | 100 | 100 | 100 | 100 | 100 | 98 |
| BNCV | 100 | 98 | 96 | 85 | 83 | 70 | 64 | 32 |
| LbpB | 34 | 25 | 0 | 0 | 0 | 0 | 0 | 0 |
| PBS | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Sera dilution.

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

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